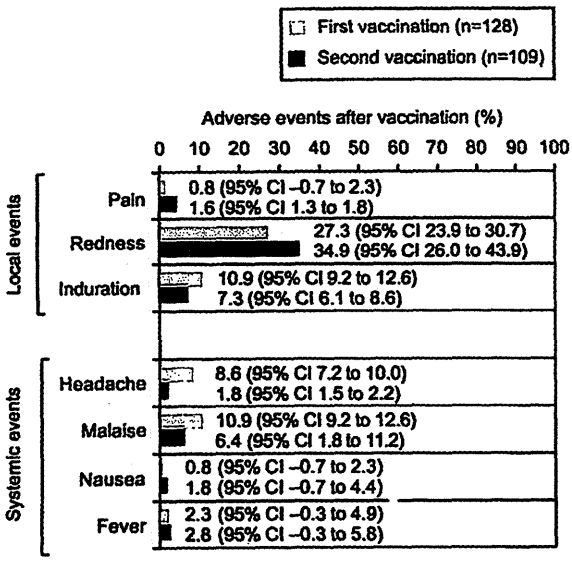

Fig. 6. Adverse reactions. The most common adverse reaction was redness at the vaccination site; the incidence of other individual adverse reactions was less than 10%. No severe adverse reaction requiring medical intervention was reported. There was no significant difference between the first vaccination and the second vaccination in terms of adverse events. CI, confidence interval.

Horiya. H1N1 Vaccination During Pregnancy. Obstet Gynecol 2011.

the gestational stages of vaccination (P=.488), and the number of occurrences was proportional to the number of participants vaccinated during each stage of gestation. The total incidence was 4.2%, which is within the range of spontaneous incidence.[29,30] In addition, because there have been no reports of specific malformation(s) related to seasonal influenza vaccines, it is unlikely that any of the cases of malformation was related to vaccination.

DISCUSSION

Because pregnant women have a high risk of being infected with and having morbidity from influenza, vaccination with an inactivated influenza vaccine during pregnancy is recommended. Although the efficacy of vaccination has been extensively evaluated, it is difficult clinically to assess the acquisition of immunity because of the presence of pre-existing antibodies, or in epidemiologic surveys because of differences between the vaccine strains and the post-vaccination epidemic viral strain. Vaccination against the 2009 H1N1 virus had the following characteristics: those vaccinated had no pre-existing antibodies because it was a new type of virus; because the viral strain that might cause a pandemic was predicted, the vaccine strain that could intercept infection was predicted accurately and the vaccine was manufactured before the epidemic; only one viral strain was used in the vaccine.

In the present study, the prevaccination antibody positivity rate (pre-existing antibodies) was as low as 7.2% because vaccination was initiated before the epidemic. After the second vaccination, the antibody positivity rate increased to 89.5%, which was consistent with approximately 90% in our previous study of seasonal influenza vaccination in pregnant women.[24] In addition, the vaccine produced a sufficient immune response regardless of the alterations in immunity as classified by Th1/Th2 ratio or stage of gestation. Vaccination was immunologically useful in all stages of gestation.

As for the vaccination frequency, 87.9% of participants (95% confidence interval 82.2–93.6) developed antibodies after the first vaccination, with a seroconversion rate of 81.5% (95% confidence interval 74.7–88.3) and change in antibody titers of 7.6-fold (data not shown). These numbers meet the

Table 4. Vaccination Timing and Outcomes

	Trimester			
	First (n=15)	Second (n=74)	Third (n=29)	All (n=118)
Maternal				
Abortion	0	—	—	0
Preterm	6.7	1.4	6.9	4.2
Gestational age (wk)	38.5±1.6	38.7±1.6	38.8±1.5	38.7±1.6
Neonatal				
Malformation (case)	0 (0%)	3 (4.1%)	2 (6.9%)	5 (4.2%)
Apgar score less than 7 at 5 min (case)	0 (0%)	1 (1.4%)	0 (0%)	1 (0.8%)
Birth weight (g)	3,025.1±407.5	3,034.0±453.2	3,026.3±394.8	3,035.3±430.6
Actual weight/median weight (%)	103.3±16.2	98.7±12.0	98.3±12.3	99.2±12.7

Data are % or mean±standard deviation.

criteria (more than 70%, more than 40%, and more than 2.5, respectively) specified by the European Medicines Evaluation Agency. Single vaccination is thus considered sufficiently potent, whereas subsequent maintenance of adequate antibody titers until delivery and antibody transfer to the newborn are also important.

In this study, second vaccination did not significantly increase the percentage of responsive participants, although it did increase antibody titers in 27% of participants. When the participants were classified by the time from vaccination to delivery, antibody titers in maternal blood at the time of delivery tended to be higher in those who received double vaccination. Among participants who received vaccination within 10 weeks of delivery, transfer to umbilical cord blood was higher in those who received double vaccination than in those who received single vaccination. However, differences were not statistically significant, suggesting that single vaccination is useful even in the absence of pre-existing antibodies and when considering maintenance of antibody titers and antibody transfer to the fetus. The optimal vaccination frequency may have to be determined based on factors such as specific epidemiologic data, the cost, labor, and adherence.

The overall incidence of adverse reactions was low, less than 10% for all adverse reactions except for redness, because the vaccine is a split vaccine containing no adjuvant. In participants who received double 2009 H1N1 vaccination during pregnancy, adverse reactions were not markedly augmented or attenuated by the second vaccination. Moreover, early delivery or abortion, malformation, and birth weight were not significantly affected. Nonetheless, the sample size was insufficient to fully evaluate the safety of the vaccine; additional information from larger studies is needed to determine this.

Historically, there have been few opportunities to evaluate an influenza vaccine under conditions where those vaccinated had no pre-existing antibodies, and the vaccine strain corresponded perfectly to the epidemic strain. Thus our results may provide valuable information. We hope that, complementary to epidemiologic assessments, this immunologic assessment will be helpful in discussions of countermeasures against a possible outbreak of highly pathogenic influenza viruses.

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妊娠中の薬剤使用とリスク評価
—妊娠と薬情報センターの役割—

村島温子*

妊娠中の薬剤使用は慎重にすべきなのは当然のことである。しかし，妊娠していると知らずにこれらに曝露された場合や，診療のために薬剤の使用が必要なときには単に怖がるだけでなく，これらが胎児にどのような影響を与えるのかを正しく理解して対応すべきである。妊娠中に薬剤を使用するかしないかは，使用するリスクと使用しないリスクのバランスで判断すべきであるが，リスクの評価のもととなる情報が極めて少ないのが現状である。このような問題を解決するために開設された「妊娠と薬情報センター」を上手に利用していただきたい。

はじめに

薬剤を投与すべきかどうかは，その効果とリスクのバランスをみて判断される。妊婦・授乳婦に対しても「リスクを考慮しても薬剤を投与することにより得られる効果が病態の改善にとって必要である」と判断したときにのみ処方するという点では同様である。妊婦・授乳婦で特別なのは，妊婦・授乳婦へ薬剤投与を行うことにより薬剤を必要としていない胎児・乳児にも薬剤が投与されることである。すなわち，児にとっては副作用のリスクのみが負荷されることになるわけで，これが特に慎重さが求められる所以である。また，流産や児の先天異常は原因がなくても発生するものである。このような症例でたまたま妊娠中に薬剤を使用していた場合はその薬剤が原因と思われてしまいがちである。母親や肉親が後悔をずっと引きずるような不幸を避けるためにも妊娠中の薬剤使用は慎重にしたいところである。一方で，母体管理に必要な薬剤もあるので，安全に関する確かな情報をもとに適切な対応が望まれる。

1．妊娠中の薬剤使用における安全性の考え方

まず，われわれから「妊娠中の薬物使用＝奇形」という先入観を取り払う必要がある。先天異常（奇形）の発生率は全分娩のうち約3%前後であり，そのほとんどが薬剤とは関連していない。すなわち，薬剤を使用しようとしなかろうと3%前後に先天異常は発生している事実を医師も患者も知っておくべきである。ちなみに自然流産の自然発生率も15%前後ある。奇形全体のうち薬剤が原因とされる奇形はわずか1%以下である。この1%以下というなかに，抗てんかん薬のようにリスクが明らかでも服用しながら妊娠を継続せざるをえないケースも含まれているということを考えると，薬剤が原因の先天異常がいかに少ないかがわかる。また，リスクのある薬剤を使用したまま妊娠してしまった場合にも，10%を超えて先天異常を生ずる薬剤

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表1 催奇形性の確率による分類（疫学研究をもとにした）

	薬剤の種類ないしは一般名	
高リスク（>25%）	サリドマイド 男性ホルモン 蛋白同化ステロイド	
中等度のリスク（10~25%）	ワルファリン ビタミンA誘導体 D-ペニシラミン	
低リスク（<10%）	抗てんかん薬	バルプロ酸 カルバマゼピン フェニトイン フェノバルビタール プリミドン
	抗腫瘍薬 メソトレキセート ミロプロストール チアマゾール リチウム	

（文献1より改変）

はわずかであり[1]（表1），われわれは安易な中絶に導かないようにしなければならない。

われわれ医師が薬を投与する際には，添付文書を参考にするのは妊娠中も非妊娠時にも変わりはない。添付文書は薬剤が治験により安全性，有効性を確認され，発売される際に作成される。しかし，妊婦を対象とした治験は倫理上不可能であり，発売される段階で持ちうる妊娠中の使用に関する安全情報は動物実験結果のみである。動物実験をそのままヒトに適用する（外挿）ことには限界がある。奇形発生の動物とヒトとの一致率をマウス，ラット，ウサギ，ハムスター，サルでみた研究結果がある。これでは，ヒトに対する催奇形性物質の動物における偽陰性は3%，ヒトに対する非催奇形性物質の動物における擬陽性は72%であった[2]。したがって，添付文書で「有益性投与」であっても，発売直後の薬剤の妊娠中の使用は控えるのが無難である。一方で，動物実験を根拠にいつまでも妊婦禁忌の薬剤が存在する。「ドンペリドン」は制吐薬であり，つわりと知らずに嘔気を主訴に受診し，処方されている例が多いことは容易に想像できるが，これまでその催奇形を疑うような報告はない。すなわち経験的にリスクはないものと考えられるが，添付文書では妊婦禁忌のままであり，それをネットなどで知った女性が不安になるのも仕方ない。添付文書は，危険度を上げる際には俊敏に動くが，最近，禁忌から有益性投与になったラベタロール，ニフェジピン（20週未満は禁忌のまま）を除くと安全性を上げる方向へ変更されることは皆無である。このような背景を知った上で，妊娠中の薬剤使用に関する評価やカウンセリングをしていかなくてはいけない。

このように動物実験結果がそのままヒトに適用できるわけではないので，ヒトで使った場合に安全かどうかは使用経験から判断するしかない。当然ながら，発売後すぐに出てくるヒトでの使用経験は症例報告であるが，われわれは悪い結果のほうが報告されがちであることを念頭に解釈しなければならない。臨床的に重要な薬剤では研究者や製薬会社などによって前向きコホート研究が行われる場合があるが，コストや時間など大きな労力がかかる。一方，北欧の

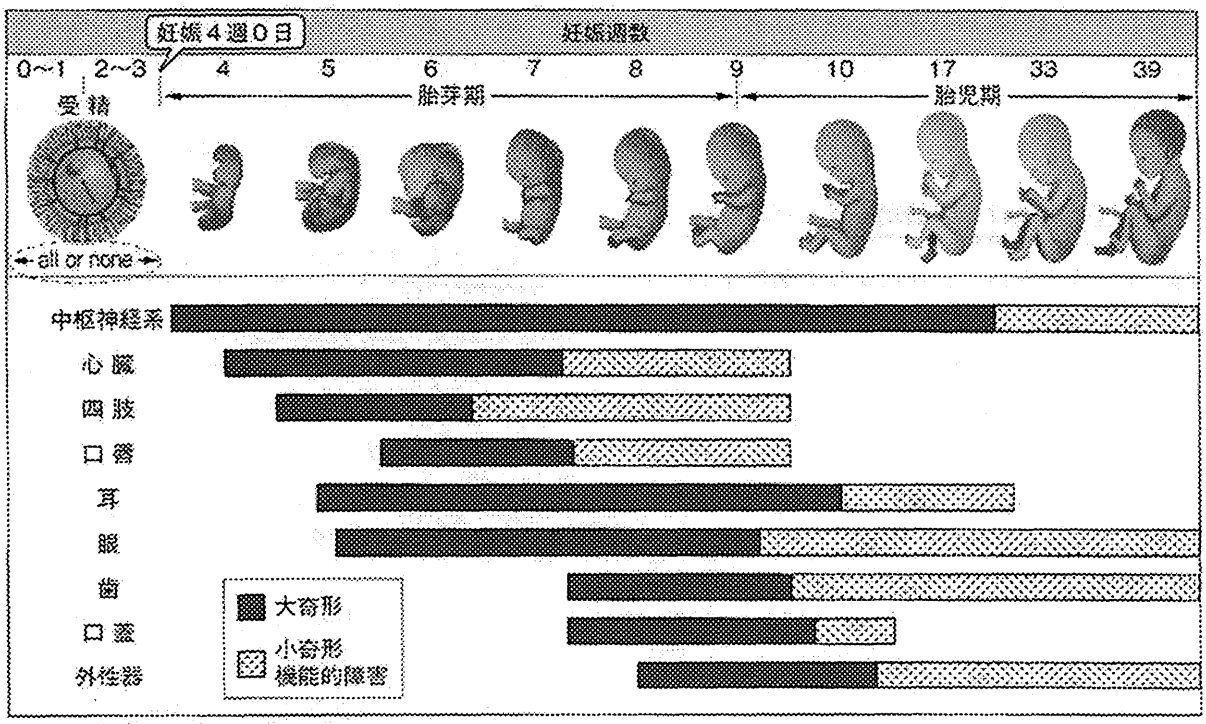

図1 胎児の発生における危険期

国々のように患者登録制度が充実しているところでは，これを利用した前向きコホート研究結果が次々と発表されている。しかし，これらは実際に服用したかどうかの確認が取れていないなど，その精度について危惧する声もある。

安全性の判断資料として，日本の臨床現場では添付文書の記載以外に米国医薬品局（FDA）の分類が重宝されてきた。しかし，これは疫学研究，動物実験，臨床的有用性でランク付けされたもので，合理性に欠けるとの判断から3年前，分類は廃止され，記述式で表示されることに決定した。

2．妊娠時期と児への影響（図1）

受精からおよそ2週（14＋α日）間は受精卵が薬剤や放射線から障害を受けた場合流産となる。逆に流産にならなかった場合には奇形の形で影響が残ることはないと考えられている。この期間は「All or None（全か無か）」の時期と呼ばれる[3]。その後～7週は重要臓器が形成される時期で奇形の絶対過敏期となる。妊娠8～12週は口蓋や外性器がつくられており，まだ慎重な対応を要する。

表2 胎児毒性のリスクのある主な薬剤

薬剤の種類	症候
アルコール	胎児アルコール症候群
NSAIDs	動脈管早期閉鎖による肺高血圧症，羊水減少，分娩遷延
ACE阻害薬	胎児の低血圧と腎血流低下による頭蓋冠低形成や腎機能異常
AⅡ拮抗薬	胎児の低血圧と腎血流低下による頭蓋冠低形成や腎機能異常
抗甲状腺剤	甲状腺機能低下，甲状腺腫
ヨード（大量）	甲状腺機能低下，甲状腺腫
精神系薬剤	出生児の呼吸障害，出生後しばらくしての離脱症状

NSAIDs：非ステロイド性抗炎症薬
AⅡ受容体拮抗薬：アンギオテンシンⅡ受容体拮抗薬

妊娠中期以降は奇形の心配がなくなるからといって，何でも投与できるわけではない。胎児毒性という観点からの考慮も不可欠である。胎児毒性のある主な薬剤を表2に示す。胎児毒性が明らかな薬剤は避けるのはもちろんであるが，胎児毒性を証明することは難しいため，胎

盤移行性の高い薬剤の安易な投与は避けるべきである。また，同じ薬効であれば胎盤移行性の低い薬剤を，新生児にも使用される薬剤を選ぶべきであろう。

3．生殖年齢女性への薬剤投与の基本姿勢

1 慢性疾患を持つ女性に対して薬剤を投与する場合

非妊娠時には慢性疾患の管理が優先されるが，可能であれば，いつ妊娠してもよいような薬剤選択を行う。妊娠初期の薬剤使用は児の奇形につながるとして，必要な薬剤であっても妊娠したとたんに中止する例をしばしば見かけるが，妊娠を継続させ，母児ともにベストな状態で出産に持っていくためには薬剤で原疾患をしっかりコントロールする必要があることを説明し，理解してもらっておくことが肝要である。また，疫学研究でリスクが否定されているが添付文書上「妊婦禁忌」である薬剤，あるいはリスクがあるとわかっている薬剤であっても，疾患のコントロールと妊娠の両立のために使用しなければならない場合もある。前者の場合には，リスクが否定されている根拠をわかりやすく説明し，患者の同意をとれば使用できると考える。後者のうち，ワルファリンのように妊娠が判明してから中止するしかない薬剤では「全か無か」説（前述）を利用した計画妊娠を行う。また，抗てんかん薬のように児へのリスクがあっても妊娠中も継続しなければならない場合には継続した場合のリスクと中止した場合のリスクについて具体的にわかりやすく説明し，安易な中止や中絶を避けるような働きかけが必要である。

また，薬剤のなかには卵巣機能に影響を与えるものもあり，生殖年齢の女性に薬剤を投与する際には妊孕性に対しても注意を払う必要がある。最も有名な薬剤は抗がん剤，免疫抑制剤として使用されるシクロフォスファミドである。

2 妊娠可能年齢の一般女性，妊婦に対して薬剤を投与する場合

妊娠する可能性のある女性には「より安全」で，添付文書で「禁忌」になっていない薬剤を投与するのが無難である。疫学研究でリスクが否定されている，古くから頻用されてきているということが「より安全」と判断する根拠である。

4．妊娠と薬情報センター[4]について

1 設立の目的[5]

妊娠と薬情報センターは妊娠中の薬剤使用に関する情報を提供するとともに，妊娠中に薬物使用した症例の妊娠転帰を集積し，エビデンスを創出していくことを目的として設立された。

2 設立に至った背景

国立成育医療研究センター（以下，当院）は，胎児から小児，思春期を経て出産に至るまでのリプロダクションサイクルを対象とした総合的かつ継続的医療の推進を目的に2002年にオープンしたナショナルセンターである。当院では従来の産婦人科のほかに，胎児診療科，不妊・不育診療科，母性内科，新生児科が情報を共有し，リプロダクションに関するチーム医療を行っている。当然のこととして，慢性疾患を持つ女性の妊娠，すなわち合併症妊娠や，妊娠中に偶発的に出現する妊娠高血圧症候群などの妊娠合併症を診療する機会が多く，妊娠中の薬剤使用の安全性は大変重要なテーマである。各科の医師と薬剤部など関連するスタッフが，カンファランスなどを通して情報を吟味し，共有するという活動が自然発生的に生まれ，2003年12月の「妊娠・授乳と薬相談外来」開設に発展し，院内患者のみならず院外の女性にも情報を提供することとなった。一方で，行政側も妊娠中の薬剤使用に関する情報の提供，疫学データの構築の必要性を認識し，厚生労働省の事業として「妊娠と薬情報センター」設立が決定した（図2）。このように，臨床の現場と行政が同じ方向を向いた取り組みを同時に始めていたという偶然も手伝って，当院に「妊娠と薬情報センター」が置かれたのである。

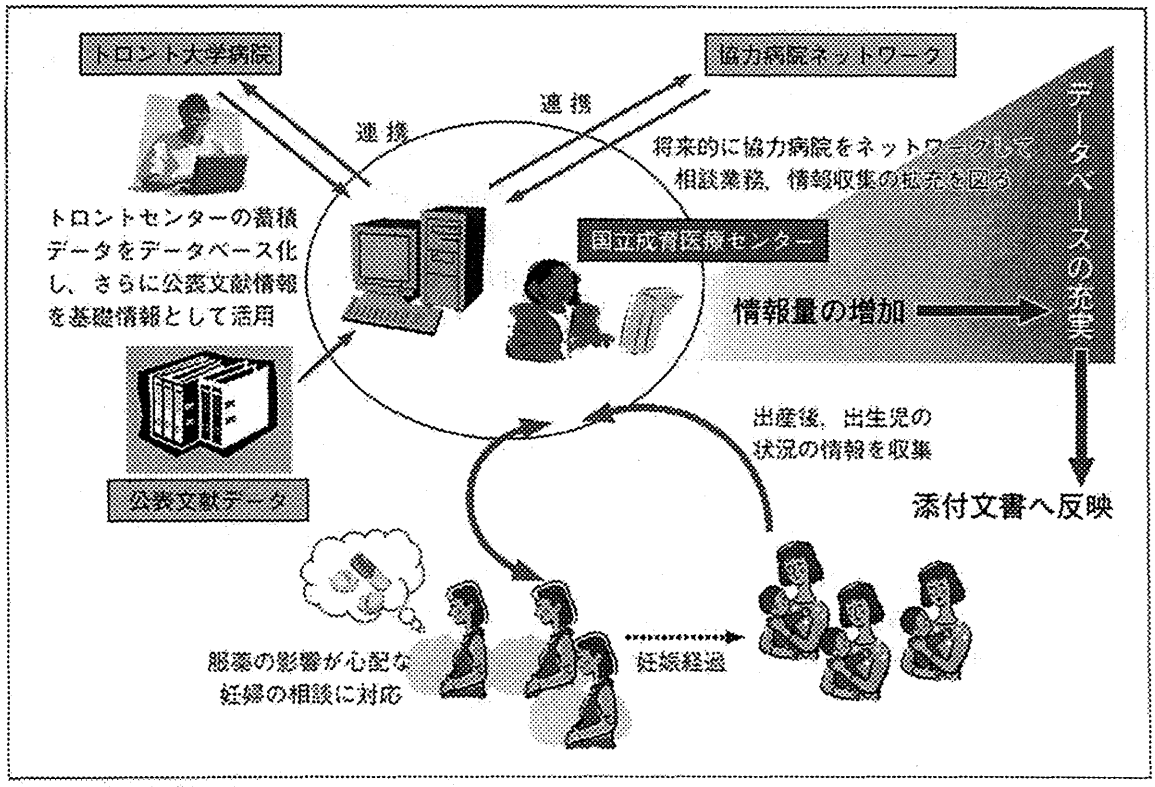

図2 妊娠と薬情報センター

3 開設準備について

1. 厚生労働省主催の検討会での議論

この領域の専門家，法律の専門家などにより構成された検討会が開催され，当センター事業のあり方について3回にわたり活発な議論が交わされた。

第一回検討会ではバックグラウンドの把握のために，この事業の簡単な紹介の後，虎の門病院，聖路加病院で行われてきている「妊娠と薬」外来の報告と当院で行っていた外来の説明，日本産婦人科医会・先天異常モニタリング（本部：横浜市立大学医学部産婦人科）についての説明が行われた。第二回検討会では相談者に提供する情報の作成方法および情報提供の方法についての検討が行われた。第三回検討会では第二回検討会における情報提供の方法についての意見を踏まえ再考した結果を提示し，最終的な合意を得た。さらに相談例のフォローアップに関し，同意のとり方を中心に検討が行われ，運用方針が決定された。

2. 海外の奇形情報サービス（teratology information service）との連携

開設準備の段階から，この領域の世界的リーダーであるカナダトロント小児病院のマザーリスプログラム（MRP）[6]により強力なバックアップをいただいている。MRPは，トロント小児病院臨床薬理部のスタッフであるGideon Korenにより，若干の研究費をもとに1985年に創設された。電話を中心とした妊婦・授乳婦の薬剤使用に関するカウンセリングを行うとともに，相談例の妊娠転帰のフォローアップによるコホート研究を並行して行って，数多くのエビデンスを創出してきた。現在，MRPとは相談者に提供する薬剤の安全性情報の交換を行い，その質管理を図るとともに，カウンセリングの質向上を目指して当センターの実務担当者をMRPに派遣し，研修をお願いしている。MRPで研修して自国へ戻った外国人留学生のつながりのもとに，マザーリスクプログラム・リサーチネットワークが動き出しているが，当院はトロント小児病

院と正式な業務提携の合意に達し，当センターはこのリサーチネットワークの重要なメンバーとなった。

MRPのほかに，OTIS（北米TISネットワーク），ENTIS（ヨーロッパTISネットワーク）とのネットワークも構築されつつある。

4 妊娠と薬情報センターの業務

1. 妊娠と薬情報センターの組織

産科，内科，新生児科（小児科）医師および薬剤師を実務スタッフとし，薬理，遺伝，生命倫理などの当院内の専門家がアドバイザーとして加わっている。さらに，提供する情報の正確性を担保するために，外部委員も含めた成育ステートメント検討委員会が設置され，定期的に開催されている。この検討委員会では新情報が出された場合（例：パロキセチンと心奇形・新生児肺高血圧症との関係）の解釈をどのように行うか，タミフル®などの情報がほとんどないような薬剤に関する成育サマリーの文言をどのようにするかなどについて議論され，当センターが提供する情報の質の担保に大きな役割を果たしている。

2. 提供する情報の作成

妊娠中に薬剤を服用することの安全性について臨床試験を行うことは困難であり，使用経験をもとに解析したデータを参考にすることになる。実際には，効率を考えてBriggs，Micromedoxなどの二次情報データベースから情報を収集する。これら二次情報データベースの欠点はリアルタイムでアップデートできていないことである。そのため，相談があるたびにMedlineや製薬会社発表の情報をチェックし，採用すべきものがあれば追加している。採用すべきかどうか判断が難しい情報については前述の検討委員会で議論するとともに，前述のMRPやOTISやENTISなどからの情報も参考にして，最終決定を行っている。

日本で開発された薬剤の多くは妊娠中の使用に関する疫学データをほとんど持っていない。このような場合の情報源はインタビューフォームに記載された動物実験結果，製薬会社が把握している症例のデータだけとなるため，これらの解釈が重要となる。しかし，これらの解釈は非常に難しいため，やはり専門家のそろう検討委員会で検討し，質の担保を図っている。

3. 相談の実際

1）相談方法

相談手順を図3に示す。当センターのホームページからダウンロードした（できない場合には当センターから郵送も可）問診票に必要事項を記入し郵送することから相談が始まる。申し込みは患者本人であることを原則としている。この申し込みの段階で3通りの方法のうちから希望する方法を選んでもらう。問診票を受け取った当センターの事務局は，相談者の希望と相談内容を勘案して相談方法を決定し，その旨を通知する。

相談方法は現在のところ3通りある。当センターの委託を受けた拠点病院の「妊娠と薬外来」での相談，主治医のもとでの相談（当センターから送付された回答書を用いた），電話相談（当センタースタッフによる）である。リスクの低い薬剤については主治医ないしは電話での相談も可能であるが，主治医から説明を受けたい場合には，問診票を送付する際に相談依頼書（通常の紹介状でも可）の同封が必要である。抗てんかん薬のような催奇形性が明らかな薬剤の場合や妊婦の不安が強い場合などでは修練した専門家からの説明が必要であるため，基本的に「妊娠と薬外来」で行うことにしている。拠点病院は2010年3月現在，19カ所である。拠点病院の担当医師・薬剤師を対象に研修を行い，情報や知識の共有，カウンセリング手法の修練に努めている。今後も拠点病院を拡充し，相談者の利便性を向上させていきたいと考えている。

2）各カウンセリング方法の長所・短所

拠点病院の「妊娠と薬外来」では修練した医師と薬剤師の連携のもとにカウンセリングを行っており，質が担保されているが，施設数に限りがあるのでアクセスが悪い相談者も多いであろう。また，カウンセリングであるため，有料である（自費診療としているが，料金はその

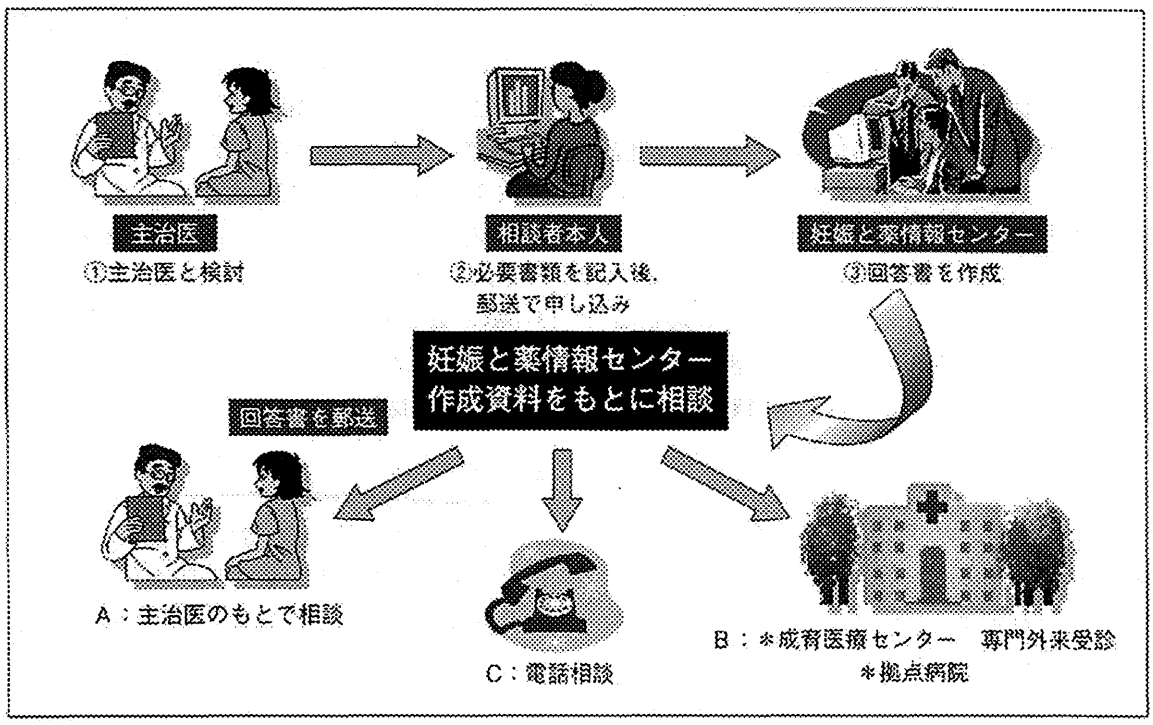

図3 相談手順

施設に任せている)。主治医からの説明はアクセスという点，ならびに薬を使用する状況を知っているという長所はあるが，当該分野に不慣れで質のよいカウンセリングができない可能性がある。電話による相談は，簡便かつ無料(電話代のみ)で質のよいカウンセリングができるという点ではよいが，カウンセリングをする立場からは，顔の表情が見えないので理解してもらえたどうかの判断ができないのが難点である。

3) 授乳に関する相談

当センターを除き，授乳と薬剤に関する相談は受けていない。その代わり，ホームページに基本的考え方ならびに授乳と両立ができる薬剤と避けるべき薬剤について表にまとめて公開している。

4. 疫学研究の方法

当センターの相談業務に必要な問診票に記入された情報と分娩後に送付されてきた妊娠結果調査ハガキに記入された情報を，電子化して保管し，ある程度の数が集まった薬剤，緊急性のある薬剤(タミフル®など)を対象とし疫学研究を行い，その結果を公表することにしている。また，前述したMRPネットワークならびにOTISやENTISなどの世界の情報ネットワークに参加し，当該領域の研究で世界に貢献したいと考えている。

1) 研究協力の説明

本事業は相談者に妊娠中の服薬に関する最新の情報を提供するとともに，その結果を調査し，将来の相談事業に役立てていくという2つの目的があることを，相談者に説明し，同意を得た上で実効性のあるフォローの方法をとっていくべきというスタンスをとっている。

5. 妊娠結果の調査

本事業の大きな柱である疫学研究を行うためには妊娠結果のデータ取得は必須である。相談時に妊娠していて，かつ研究協力について同意している例を対象に分娩予定日の1カ月後ぐらいをめどに妊娠結果調査ハガキを送付し，必要事項を記入して返送してもらっている。

5 妊娠と薬情報センターの現状と展望

1. 相談の実績

相談数はオープンしてからずっと増加してきている。相談薬のうち，うつ，てんかんなど精

神・神経疾患に処方された薬剤が半分近くを占め，感冒・インフルエンザ関係薬剤，アレルギー薬が後に続く。また，当センターの存在が医療従事者の間に知られるに従い，精神・神経疾患以外の慢性疾患で用いられる薬剤も少しずつ増えてきている。

2. 教育，広報活動

毎年，数カ所ずつ新規拠点病院に「妊娠と薬外来」が開設されるのに合わせ，拠点病院の担当医師および薬剤師を対象に業務研修会を開催している。ここでは奇形学，動物実験学，薬理学，合併症妊娠管理など関連する分野の第一人者を講師としてお呼びし，実技を含めて3日間の研修を行っている。

また，秋の開設記念日に合わせて講演会（妊娠と薬情報センター開設○周年フォーラム）を開催している。コンセプトは「薬物治療の進歩に妊娠と薬情報センターはどうキャッチアップしていくか」であり，2010年度は腎・高血圧と呼吸器疾患治療薬を対象に開催し，好評を博した。2011年度は精神神経疾患治療薬を対象にして行う予定である。

3. 疫学研究体制の整備

当センターで追跡調査の対象として重視しているのは日本で開発された薬剤である。これらの多くは疫学研究のデータを持っていない。その代表的な薬剤であるエチゾラム（デパス®），ロキソプロフェン（ロキソニン®）は，相談数が多く，近い将来エビデンスが出せるものと考えている。

緊急性の高い薬剤については十分な例数が集まらなくても発表する場合がある。2009年の新型インフルエンザの流行時には，オセタミビル（タミフル®）に関して当センターのデータベースと虎の門病院のデータベースを合わせて解析し，世界で初めてのエビデンスとすることができた[7]。

慢性疾患で服用している薬剤を妊娠中にどうするかは主治医との間で完結している場合が多く，当センターに相談してくることは多くないものと思われる。すなわちこのような薬剤についてはこちらから積極的にデータベースへ組み込んでいかなくてはならない。現在，当センターをレジストリシステムとして利用し，疫学研究を行うプロジェクト（Pregnancy Outcomes of Exposure to Methimazole：POEM Study）が進行中である。今後も学会や研究会とタイアップして特定薬剤のレジストリ調査を行っていくつもりである。

おわりに

この分野は産科医，内科医，小児科医などの医師，薬剤師など多分野の専門家がかかわらなければ発展していかないと実感している。臨床と行政の熱意で誕生し，多くの方々のご理解とご協力でここまで成長した「妊娠と薬情報センター」であるが，今後はこの分野の中心となってその発展に貢献していきたいと考えている。皆様のご指導・ご支援をお願いしたい。

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LETTERS TO THE EDITOR

Characteristics of pregnant Japanese women who required hospitalization for treatment of pandemic (H1N1) 2009 - low mortality rate may be due to early antiviral use

Although evidence from several countries demonstrated increased hospitalization rates and disproportionately higher rate of mortality in pregnant women,[1–3] no maternal mortality from pandemic (H1N1) 2009 had occurred in Japan according to the information released by the Japanese Ministry of Health, Labour and Welfare (JMHLW).[4,5]

On July 2010, we requested 2611 obstetrical facilities that are members of Japan Association of Obstetricians and Gynecologists (JAOG) to provide information concerning pregnant women who were admitted to the hospital for treatment of pandemic (H1N1) 2009 during May 2009 and March 2010. A total of 2082 (79.7%) of 2611 obstetrical facilities responded and information on a total of 181 hospitalized women was provided from 126 facilities. A total of 738,766 women gave birth at these 2082 facilities, accounting for approximately 75% of all deliveries that occurred in Japan during the study period of 11 months. Seventeen of these (9.4%) developed viral pneumonitis, 2 of whom required admission to an ICU. However, all 181 women recovered completely. Maternal age was not associated with the risk of hospital admission for treatment of pandemic (H1N1) 2009. Multiparous women accounted for 52.7% of all women who gave birth in national data of Japan in 2008; whereas multiparous women accounted for 66.9% of the 181 women who required hospitalization and 76.5% of 17 women who developed pneumonitis. Women in the third trimester accounted for 74.6% (132/177) of the hospitalized women. Thus, both multiparous women and women in the third trimester were significantly more likely to require hospitalization than those without these characteristics ($p < 0.001$).

Information on the use of antiviral drugs such as oseltamivir and zanamivir was available for 118 women (Table 1); 114 (96.6%) of these women took these drugs. Treatment with antiviral drugs was initiated early (within 48 h after symptom onset) or later (more than 48 h after symptom onset) in 105 and 9 women, respectively. Six of 105 women (5.7%) who received early treatment and 3 of 9 women (33.3%) who received later treatment developed pneumonitis. Thus, women who received later treatment were more likely to develop pneumonitis than those who received early treatment. Vaccine for pandemic (H1N1) 2009 became available after mid-November in Japan. Eighty-nine (49.2%) of 181 women contracted flu in and after December 2009. The status of vaccination for pandemic (H1N1) 2009 was specified for 57 of these women, 50 of these women (87.7%) were not vaccinated. The progression rate to pneumonitis did not differ significantly between the 7 vaccinated women and the 50 unvaccinated women (Table 1). Abortion at <22 weeks of gestation occurred in 3 (1.7%) women (Table 2). A total of 178 infants were born alive to 178 women at ≧22 weeks of gestation. In 2008, 5.8% of pregnant women (62,808/1,091,156) had a preterm birth, whereas 14.6% of women who required hospitalization (26/178) had a preterm birth. Thus, women who required hospitalization were more likely to give birth prematurely (RR, 2.5; 95% CI, 1.7–3.6) than those in the general population (Table 2).

Our results indicated that at least 181 pregnant women required hospitalization for treatment of pandemic (H1N1) 2009. Since Japan has approximately 1,080,000 births (maternity) annually, these findings suggest that approximately 1 in 6000 pregnant women required hospitalization for treatment of pandemic (H1N1) 2009. According to the information released by JMHLW, a total of 17,646 persons were hospitalized for treatment of pandemic (H1N1) 2009 in Japan,[5] which represented 0.014% of the total population of 127 million, i.e., 1 in 7200 Japanese required hospitalization. Thus, pregnant Japanese women did not have an increased risk of severe complications, inconsistent with

Table 1 Effect of antiviral agents and vaccination on the progression to pneumonitis.

	Pneumonitis		Statistical analysis
	Absent	Present	
Use of antiviral agents[a]			
No. of women	164	17	RR (95%CI)
≤48 h after onset	99	6	Reference, 1.0
>48 h after onset	6	3	5.83 (1.74–19.5)
No antiviral drugs	3	1	4.38 (0.68–28.3)
Unkown/missing	56	7	
Vaccination†			
No. of women	82	7	χ^2 test
Yes	7	0	$p = 0.99$
No	48	2	
Unknown/missing	27	5	

† Vaccine for pandemic (H1N1) 2009 became available after mid-November 2009 in Japan.

[a] Oseltamivir or zanamivir. RR, relative risk; CI, confidence interval.

Table 2 Risk of preterm birth/abortion among 181 women who needed hospitalization.

Characteristics	Japan[a]	Overall	Pneumonitis	
			Absent	Present
No. of women	1,091,156	181	164	17
Abortion at <22 weeks	NA	3/181 (1.7)	2/164 (1.2)	1/17 (5.9)
Preterm birth				
22–31 weeks	7876/1,091,156 (0.7)	5/178 (2.8)†	5/162 (3.1)†	0/16 (0)
32–36 weeks	54,932/1,091,156 (5.0)	21/178 (11.8)†	16/162 (9.8)†	5/16 (29.4)†‡
Term birth	1,028,348/1,091,156 (94.2)	152/178 (85.4)†	141/162 (86.0)†	11/16 (68.8)†

NA, National statistics concerning spontaneous abortion at <22 weeks of gestation is not available.
† $p < 0.01$ vs Japan (national statistics); ‡ $p < 0.05$ vs women group without pneumonitis.
[a] National data of Japan in 2008 were presented as a comparison group.

those in other countries.[1–3] One possible explanation for the difference is the frequency of the antiviral drug use. In this study, 114 (96.6%) women took antiviral drugs and 105 (89.0%) took antiviral drugs early (Table 1). The rate of early antiviral drug use was much lower (43%) among pregnant women admitted to ICU in the US where at least 56 pregnant women died.[2] Furthermore, the RR of developing pneumonitis was 5.8 times higher for women who took antiviral drugs later compared to women who took antiviral drugs early in this study (Table 1). Thus, the higher frequency of the antiviral drug use may have contributed to the smaller number of pregnant patients who required hospitalization and to the lack of maternal mortality in Japan.

In conclusion, the present study indicated that the rate of admission to the hospital for pandemic (H1N1) 2009 among pregnant women was nearly equal to that for the general Japanese populations. The rate of spontaneous abortion was not higher but the risk of preterm birth was 2.5 times higher for women who required hospitalization for the treatment of pandemic (H1N1) 2009 compared with the pregnant women in the general population. Of the women who required hospitalization, those who received later treatment with antiviral drugs were 5.8 times more likely to develop pneumonitis than those who received early treatment (within 48 h after symptom onset). A higher frequency of the antiviral drug use may have contributed to the lack of maternal mortality due to pandemic (H1N1) 2009 in Japan.

Contribution

(1) Conception and design; A.N., H.M., N.U., S.S.
(2) Acquisition of data; A.N., S.S.
(3) Analysis and Interpretation of data; A.N., H.M., N.U., S.S., M.M.
(4) Drafting article; A.N., H.M., M.M.
(5) Final approval of the version to be submitted; Y.Y., T.T.

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Letter to the Editor

Impaired glucose tolerance during pregnancy: Possible risk factor for vaginal/anorectal colonization by Group B *Streptococcus*

Dear Editor,

Diabetes mellitus is a risk factor for various kinds of infections. Diabetes mellitus patients are prone to develop infections during pregnancy and puerperium. In our study, we found that pregnant women with impaired glucose tolerance have a higher carrier rate of Group B *Streptococcus* (GBS) than those with normal glucose tolerance.

We retrospectively studied 5855 pregnancies of women who delivered at Tsukuba University Hospital from January 2001 to May 2010. The samples for GBS carrier screening were obtained from the perineum and rectum at 35–37 weeks of pregnancy. Prophylaxis for neonatal GBS infection was implemented according to the Centers for Disease Control (CDC) protocol. Impaired glucose tolerance included gestational diabetes mellitus (GDM) and preexisting diabetes mellitus (DM). GDM was diagnosed according to the diagnostic criteria (1995) of the Japanese Society of Obstetrics and Gynecology. The χ^2 test was used for statistical analysis.

During this period, 5379 women delivered after 35 weeks of pregnancy. From the total study population, 249 women had impaired glucose tolerance. Compared to women with normal glucose tolerance, the women with impaired glucose tolerance had a higher colonization rate (12.1% versus 16.8%; risk ratio, 1.38; 95% confidence interval, 1.04–1.84). Neonatal GBS infections were not observed. Five patients with puerperal endometritis required hospitalization; in one patient, the pregnancy was complicated with DM.

Some studies have evaluated the incidence of GBS colonization in pregnant diabetic women in the USA. Ramos *et al.* found a higher prevalence of GBS colonization in diabetic women than in non-diabetic women (43.8% versus 22.7%).[1] In contrast, Piper *et al.* reported that gestational diabetes does not alter the colonization rate of GBS (12% versus 12%).[2] In Japan, although very few studies have been performed on the prevalence of vaginal and/or anorectal GBS colonization[3] and neonatal GBS infection, the mortality of neonatal GBS infection is higher than that in theUSA.[4]

This is the first report from the Asia and Oceania region on the correlation between GDM and GBS colonization. The procedures for preventing neonatal GBS diseases should be established on the basis of the epidemiology and healthcare system of each country. Investigating the prevalence and risk factors for GBS colonization is very important for efficient prevention of neonatal GBS diseases in Japan.

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Disclosure

None declared.

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In vivo genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats

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ABSTRACT

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. In the present study, the genotoxicity of anatase TiO_2 nanoparticles was evaluated *in vivo* using the comet assay after a single or repeated intratracheal instillation in rats. The nanoparticles were instilled intratracheally at a dosage of 1.0 or 5.0 mg/kg body weight (single instillation group) and 0.2 or 1.0 mg/kg body weight once a week for 5 weeks (repeated instillation group) into male Sprague–Dawley rats. A positive control, ethyl methanesulfonate (EMS) at 500 mg/kg, was administered orally 3 h prior to dissection. Histopathologically, macrophages and neutrophils were detected in the alveolus of the lung in the 1.0 and 5.0 mg/kg TiO_2 groups. In the comet assay, there was no increase in % tail DNA in any of the TiO_2 groups. In the EMS group, there was a significant increase in % tail DNA compared with the negative control group. TiO_2 nanoparticles in the anatase crystal phase are not genotoxic following intratracheal instillation in rats.

1. Introduction

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. Based on data published in openly available scientific literature, the genotoxicity of TiO_2 nanoparticles was evaluated in *in vitro* comet assays (single-cell gel electrophoresis), bacterial and mammalian cell mutation tests, chromosomal aberration assays and *in vivo* micronucleus assays. In bacterial gene mutation assays and chromosomal aberration assays of TiO_2 nanoparticles, both negative and positive results have been reported (Lu et al., 1998; Nakagawa et al., 1997; Theogaraj et al., 2007; Wang et al., 2007; Xu et al., 2009). In the *in vitro* micronucleus assays of TiO_2 nanoparticles, negative and positive results were also reported (Gurr et al., 2005; Kang et al., 2008; Linnainmaa et al., 1997; Lu et al., 1998; Rahman et al., 2002; Vevers and Jha, 2008; Wang et al., 2007). A previous study found that TiO_2 nanoparticles generate reactive oxygen species and oxidative stress leading to genotoxicity in mammalian cells (Shukla et al., 2011). Positive results were reported in tests on DNA damage by TiO_2 nanoparticles in studies with *in vitro* comet assays (Bernardeschi et al., 2010; Dunford et al., 1997; Ghosh et al., 2010; Gopalan et al., 2009; Gurr et al., 2005; Karlsson et al., 2009; Kang et al., 2008; Nakagawa et al., 1997; Reeves et al., 2008; Tiano et al., 2010; Turkez, 2011; Vevers and Jha, 2008; Wang et al., 2007), and *in vivo* comet assays (Trouiller et al., 2009). Negative results were reported in tests on DNA damage from TiO_2 nanoparticles in studies with *in vitro* comet assays (Bhattacharya et al., 2009; Hackenberg et al., 2010; Struwe et al., 2007; Tiano et al., 2010), and *in vivo* comet assays (Landsiedel et al., 2010). In genotoxicity testing, an *in vivo* comet assay is useful for follow-up testing of positive *in vitro* findings and for the evaluation of local genotoxicity. To assess the toxicity of nanoparticles and manage their risks, it is important to understand whether nanoparticles are more toxic than micron-sized particles. Therefore, in the present study, well-dispersed TiO_2 nanoparticles of secondary sizes were studied *in vivo* comet assay using lung tissue following an intratracheal instillation to rats.

2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Shizuoka, Japan) in compliance with the Law Concerning the Protection and Control of Animals (1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (1980) and Guidelines for Animal Experimentation, Biosafety Research Center, Foods, Drugs and Pesticides. The study was performed in accordance with the ethics criteria contained in the bylaws of the Committee of the National Institute of Advanced Industrial Science and Technology (AIST).

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2.1. Chemicals

Anatase TiO_2 nanoparticles (ST-01) 5 nm in diameter were obtained from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan. As a dispersant for the particles, disodium phosphate (DSP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was applied at 2 mg/mL according to our previous study (Kobayashi et al., 2009). Ethyl methanesulfonate (EMS, Sigma–Aldrich Corporation, USA) was used as the positive control. Dulbecco's phosphate-buffered saline, regular melting agarose and Triton-X were obtained from Sigma–Aldrich Corporation, and low melting agarose was purchased from Lonza Walkersville, Inc., USA. Ethylene diamine tetra acetic acid (EDTA) disodium salt was obtained from DOJINDO LABORATORIES, Japan. Hanks' balanced salt solutions and SYBR® Gold nucleic acid gel stain were purchased from Life Technologies Corporation, USA. Dimethyl sulfoxide (DMSO), tris hydroxymethyl aminomethane and sodium *N*-lauroyl sarcosinate were obtained from Wako Pure Chemical Industries, Ltd., Japan. TE buffer solution (pH 8.0) was obtained from Nippon Gene, Japan.

2.2. Preparation and characterization of particles

In our previous study (Kobayashi et al., 2009), the DSP solution was provided as a good phosphate-buffered vehicle for preparation of the TiO_2 nanoparticles. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and agitated in an UAM015 agitating bead mill (Kotobuki Industries Co., Ltd., Tokyo, Japan) at 10–12 m/s for 2 h with 15-μm zirconium oxide (ZrO_2) beads. Subsequently, the supernatant was removed by centrifugation at 8000*g* for 1 h. TiO_2 particles in the DSP solution after sample preparation were measured by the dynamic light scattering (DLS) method (Microtrac UPA150; Nikkiso Co., Ltd., Tokyo, Japan), dropped on TEM grid and dried and then observed by transmission electron microscopy (TEM).

2.3. Animals and treatment

Sixty-four male Crl: CD (SD) rats (7 weeks old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The rats were kept individually in a positive-pressure air-conditioned unit (20–26 °C, 35–75% relative humidity) for animal housing on a 12:12-h light/dark cycle. After a 6-day acclimation, 55 rats were assigned to the study. A standard rodent pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were provided *ad libitum*.

The experimental design was decided in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). For clarifying the relationship between inflammatory response and positive findings of comet assay, the dosage was selected to induce lung inflammation or not. Based on the results of the dose-finding test and our previous study (Kobayashi et al., 2009), 5.0 mg/kg TiO_2 were used for the high dosage group which expected to induce lung inflammation, and 1.0 mg/kg were used for the low dosage group which expected to induce non-inflammation in a single instillation study. In a repeated (intermittent) instillation study, the dosage of 1.0 or 0.2 mg/kg body weight once a week for 5 weeks was selected because these dosage were expected to induce sub-acute lung inflammation or not. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and instilled in a volume of 1.0 mL/kg body weight. As a negative control, 2 mg/mL DSP was instilled intratracheally by single or repeated administration in a similar manner. EMS was used for a positive control. In our pilot study, intratracheal instillation of EMS did not shown fine results, the other side, single oral administration of EMS shown fine results in the lung epithelial comet assay. Therefore, 500 mg/kg EMS was administered orally once 3 h before sacrifice in both single and repeated study. In the single instillation group, rats were anesthetized and sacrificed 3 or 24 h after the treatment, while in the repeated instillation group, rats were anesthetized and sacrificed 3 h after the last treatment. Five rats per group, except the 0.2 mg/kg TiO_2 repeated instillation group in which one rat died, were used for each time point. The lungs were excised immediately after sacrifice. The left lobe was used for the histopathological examination, and the right lobe, for the comet assay.

2.4. Histopathological examination

The left lobes of the lungs were fixed in 10% neutral buffered formalin. All fixed tissues were routinely processed, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. The slides scored double blind.

2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the JaCVAM, as follows:

The right lobes were washed out with homogenizing buffer (Hanks' balanced salt solution containing 25 mmol/L EDTA-2Na and 10% v/v DMSO) and homogenized in about 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. The 10 μL of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide pre-coated with 1.0% agarose gel and covered with non-coated superfrosted glass. After solidification, the non-coated slide was removed, and 90 μL of low melting agarose was added again. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10 vol.% DMSO and 1 vol.% Triton X-100) for at least one night at about 4 °C in the dark. They were next placed in a submarine-type electrophoresis chamber (BIO CRAFT Co., Ltd., Tokyo, Japan) and covered with chilled electrophoresis buffer (pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was then conducted at a constant voltage of 0.7 V/cm (25 V) (current at the start: 300 mA) for 20 min. The slides were transferred into neutralization buffer and left to stand for about 10 min. Subsequently, they were dehydrated with ethanol. Finally, the slides were air-dried and stored at room temperature until scoring. The slides were stained with SYBR® Gold nucleic acid gel stain diluted 5000-fold with TE buffer. The migration of DNA in cells was examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) with IB excitation [excitation filter (BP470–490) and auxiliary absorbing filters (BA515IF)]. The final magnification was 200×. Images were taken with a CCD camera (Allied Vision Technologies GmbH, Stadtroda, Germany) attached to the microscope and analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The parameter used to measure DNA damage in the cells was the percentage of DNA in the tail [% tail DNA]. Images of 100 (50 × 2) cells per rat were analyzed. The mean % tail DNA value (mean value for 100 cells) of each group was calculated.

2.6. Statistical analysis

Data for the TiO_2 groups and negative control group were analyzed using the Dunnett multiple comparison test (two-sided,

0.05). Data for the positive control was compared to that for the negative control with Aspin–Welch's *t* test (one-sided, 0.025).

3. Results

3.1. Particle characterization

The detail of the test sample characterization was already described in our previous paper (Kobayashi et al., 2009). Therefore, we showed only the brief information about the sample characterization in the present paper. We have monitored the time-dependant change of the secondary particle size in the DSP solution from the sample preparation until the intratracheal instillation. There was no significant change in the secondary particle size during the period. There is no surface coating on the testing sample. The purity of the TiO_2 sample is 99.99%. BET surface area of the bulk TiO_2 sample is 316 mg/m^2. The secondary diameter of TiO_2 nanoparticles dispersed in 2 mg/mL DSP was 19 ± 6.7 nm (mean ± SD). The size distributions measured by DSL and TEM images of TiO_2 nanoparticles are presented in Fig. 1.

3.2. Single instillation

A single intratracheal instillation of TiO_2 nanoparticles was performed. Rats were instilled with 1.0 or 5.0 mg/kg of the particles, and euthanized and necropsied 3 or 24 h later. Clinical signs and mean body weights of all TiO_2 groups were comparable to the negative control. In the histopathological examination of the lungs (Fig. 2 and Table 1), infiltration of alveolar macrophages laden with the test compounds and/or neutrophils was observed at 24 h after treatment in the 1.0 and 5.0 mg/kg TiO_2 groups. There were no significant lesions in the lungs in the negative control, nor were there at 3 h after treatment in the 1.0 or 5.0 mg/kg TiO_2 group. In the comet assay (Fig. 3 and Table 2), % tail DNA in lung epithelial cells exposed to TiO_2 nanoparticles was comparable to that of the negative control at both 3 and 24 h. EMS, the positive control, induced significant DNA damage after 3 h exposure as compared to the negative control.

3.3. Repeated instillation

Repeated intratracheal instillation of TiO_2 nanoparticles was performed. Rats were instilled at a dosage of 0.2 or 1.0 mg/kg body weight once a week for 5 weeks. They were euthanized and necropsied 3 h after the last treatment. Clinical signs, mean body weights and mean body weight changes for all TiO_2 groups were comparable to the negative control. In the histopathological examination of the lungs, infiltration of alveolar macrophages laden with the test compounds and neutrophils was observed in the 1.0 mg/kg TiO_2 group (Fig. 2 and Table 1). There were no significant lesions in the lungs in the negative control or 0.2 mg/kg TiO_2 group. In the comet assay (Table 2), there was no significant difference in % tail DNA between the TiO_2 groups and negative control.

4. Discussion

TiO_2 nanoparticles are widely used in creams, cosmetics, pharmaceuticals and foods. Due to their photocatalytic properties, TiO_2 nanoparticles are also used as wastewater disinfectant. The respiratory tract is one of the target organs of nanomaterials when exposure occurs via inhalation. Occupational and/or environmental exposure to nanoparticles is associated with an increased risk of lung cancer. TiO_2 particles are well characterized as poorly soluble with low toxicity (Bermudez et al., 2002; Warheit et al., 2005, 2007a–c, 2008). The pulmonary effects of TiO_2 particles were observed at high doses in long-term toxicity studies in rats (Bermudez et al., 2002; Warheit et al., 1997).

One of the key disciplines governing risk assessment of substances for human health is genotoxicology due to the fact that classic genotoxic substances lead to carcinogenesis (Singh et al., 2009). Genotoxicity testing, the evaluation of the carcinogenicity and mutagenicity of substances, is the most important part of the safety testing of chemical compounds. In previous genotoxic studies on TiO_2 nanoparticles, positive and negative results were obtained *in vitro* and *in vivo*. In studies *in vitro* with the comet assay using respiratory tract organs of mammals, positive results were obtained in human bronchial epithelial cells (Gurr et al., 2005; Falck et al., 2009) and human alveolar type 2-like epithelial cells (Karlsson et al., 2009), whereas negative results were obtained in human bronchial epithelial cells (Gurr et al., 2005; Bhattacharya et al., 2009), human nasal mucosa cells (Hackenberg et al., 2010), and Chinese hamster lung fibroblasts (Landsiedel et al., 2010). In rats made to inhale a sunscreen product containing 79–89% TiO_2 nanoparticles 6 h per day for 5 days at a concentration of 10 mg/m^3, negative outcomes were obtained in the comet assay (Landsiedel et al., 2010). However, positive outcomes were obtained in comet assays using genetically modified mice exposed to TiO_2 nanoparticles (Trouiller et al., 2009). Trouiller et al. (2009) used nonstandard techniques to evaluate genotoxicity in mice fetal tissues which given poorly characterized TiO_2 nanoparticles in the drinking water to dams, and they did not measure or confirm the intake of TiO_2 particles in the mice. The inconsistencies in the results of these studies might be attributable to the differences in the test conditions, such as cell types, exposure time, concentrations, animal model, the dispersal of the particles, and the physico-chemical characteristics of TiO_2.

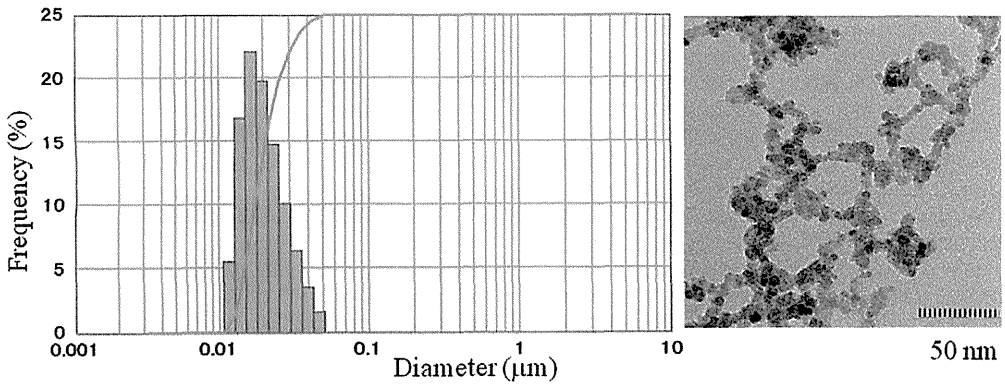

Fig. 1. Size distribution of dispersed TiO_2 nanoparticles measured by the dynamic light scattering (DLS) method and transmission electron microscopy (TEM).

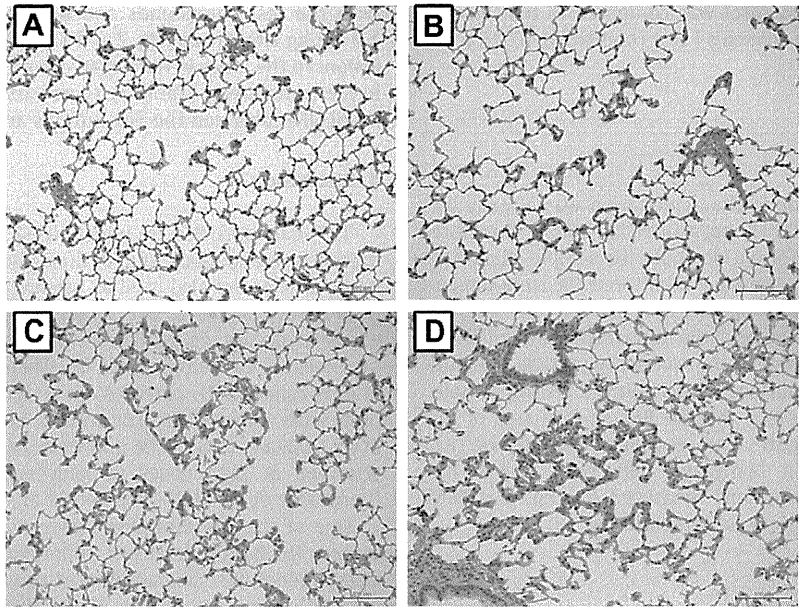

Fig. 2. Lung: (A) negative control: no significant lesions in the single instillation after 24 h; (B) single intratracheal instillation of 1.0 mg/kg after 24 h: no significant lesions; (C) single intratracheal instillation of 5.0 mg/kg after 24 h: infiltration of alveolar macrophages laden with the test compounds and neutrophils in the alveolus; (D) repeated intratracheal instillation of 1.0 mg/kg/week for 5 weeks after 3 h final dosing: deposition of test compounds, thickening of alveolar wall, infiltration of alveolar macrophages laden with the test compounds in the alveolus. H&E, bar = 100 μm.

Table 1
Histopathological findings of lung on TiO_2.

	Grade	DST	TiO_2 0.2 mg/kg	TiO_2 1.0 mg/kg	TiO_2 5.0 mg/kg
Single instillation, 3 h after dosing					
No. of rats examined		5	–	5	5
Deposition of test compounds	+	0		0	5
Infiltration of alveolar macrophages	+	0		0	1
Single instillation, 24 h after dosing					
No. of rats examined		5	–	5	5
Deposition of test compounds	+	0		5	5
Infiltration of alveolar macrophages	+	0		0	5
Infiltration of neutrophils in alveolus	+	0		1	3
Repeated (intermittent) instillation, 3 h after final dosing					
No. of rats examined		5	4	5	–
Deposition of test compounds	+	0	0	5	
Infiltration of alveolar macrophages	+	0	0	5	
Infiltration of neutrophils in alveolus	+	0	0	1	
Thickening of alveolar wall	+	0	0	1	

+: Slight.

A battery of genotoxicity assays, comprising a bacterial gene mutation assay, an *in vitro* chromosomal aberration assay, and an *in vivo* micronucleus test, serves as a reference for the type of screening information that should be addressed for new chemicals including pharmaceutical drugs (OECD, 2003; ICH guideline, 1998). The bacterial gene mutation assay and *in vitro* chromosomal aberration assay are components of the minimum base set of genotoxicity screening studies which provide a fundamental characterization of the potential hazards of nanomaterials (Warheit et al., 2007a–c). Landsiedel et al. (2010) did not find any genotoxic effects of TiO_2 products in a battery of genotoxicity assays comprising a bacterial reverse mutation assay (Ames test), micronucleus test *in vitro* in V79 cells, micronucleus test *in vivo* in mouse bone marrow cells, and comet assay *in vivo* in lung cells from rats exposed by inhalation. Anatase TiO_2 nanoparticles are known to induce oxidative stress, acellular and intracellular ROS generation, and DNA-adduct formation, but not DNA-breakage in human lung cells (Bhattacharya et al., 2009).

In conclusion, the present study clearly indicated that a single intratracheal instillation of anatase TiO_2 nanoparticles (5 mg/kg) or repeated intratracheal instillation (1 mg/kg) once a week for 5 weeks induced an inflammatory response, but not DNA damage, in the lungs in rats, therefore, TiO_2 nanoparticles in the anatase crystal phase were not genotoxic following intratracheal instillation in rats.

The *in vivo* rodent alkaline comet assay (single gel electrophoresis assay) is widely used for detecting DNA damage but has not been validated formally. Recently this assay was listed in the ICH Guidance "S2 (R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" as a second *in vivo* assay. The comet assay was recommended as a supportive study by the Guidance (Hartman et al., 2003 and Burlinson et al., 2007).

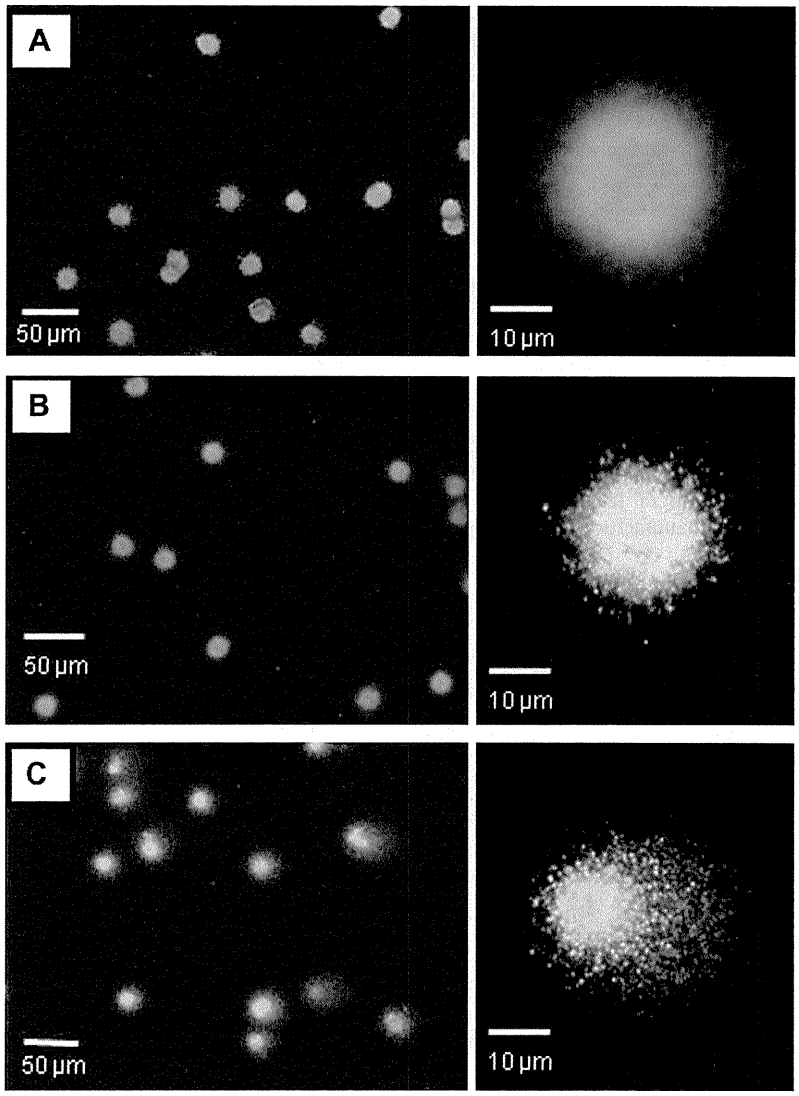

Fig. 3. Microphotographs of a comet image in the single instillation groups; (A) lung cells treated with the negative control (undamaged cell); (B) lung cells treated with 5.0 mg/kg TiO_2 (undamaged cell); (C) lung cells treated with the positive control (EMS, damaged cell). Nuclei were stained with SYBR® Gold nucleic acid gel stain.

Table 2
Results of comet assay on TiO_2.

Compound	Dose (mg/kg)	No. of rats	No. of cells analyzed	% Tail DNA (mean ± SD)
Single instillation, 3 h after dosing				
DSP	0	5	500	5.27 ± 2.63
TiO_2	1.0	5	500	3.24 ± 1.37
	5.0	5	500	5.42 ± 3.89
EMS	500	5	500	26.86 ± 6.69*
Single instillation, 24 h after dosing				
DSP	0	5	500	3.60 ± 1.37
TiO_2	1.0	5	500	2.41 ± 0.61
	5.0	5	500	1.87 ± 1.40
Repeated (intermittent) instillation, 3 h after final dosing				
DSP	0	5	500	5.64 ± 1.24
TiO_2	0.2	5	500	7.82 ± 2.11
	1.0	5	500	7.83 ± 2.23
EMS	500	5	500	29.76 ± 6.87*

DSP: disodium phosphate (negative control).
EMS: ethyl methanesulfonate (positive control).
* Significantly different from negative control at $p < 0.025$ (Aspin–Welch's t-test).

Thus JaCVAM is organizing an international validation study, in cooperation with the US NICEATM and ICCVAM, ECVAM, and JEMS/MMS. The purpose of this study is to validate the *in vivo* comet assay as an alternative follow-up assay to the more commonly used *in vivo* liver UDS assay, establishing minimal reporting standards for regulatory submissions and publications (proposed to OECD as a test guideline).

Conflict of interest statement

The authors declare that they have no conflicts of interest. The views expressed in this article are those of the authors and do not necessarily reflect the views and policies of the National Institute of Advanced Industrial Science and Technology (AIST).

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